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Title of  
Invention

PRE-SELECTION AND ISOLATION OF SINGLE NUCLEOTIDE POLYMORPHISMS

## APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO:

Assistant Commissioner for Patents  
Box Patent Application  
Washington, D.C. 20231

1. ☐ Fee Transmittal Form  
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages 40] ]  
(preferred arrangement set forth below)
- Descriptive title of the invention
  - Cross References to Related Applications
  - Statement Regarding Fed sponsored R & D
  - Reference to microfiche Appendix
  - Background of the Invention
  - Summary of the Invention
  - Brief Description of the Drawings
  - Detailed Description
  - Claim(s)
  - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 9] ]  
☒ Formal ☐ Informal
4. ☐ Oath or Declaration/POA [Total Pages ] ]
- a. ☐ Newly executed (original or copy)
  - b. ☐ Copy from a prior application (37 C.F.R. 1.63(d))  
(for continuation/divisional with Box 17 completed)  
[NOTE Box 5 below]
  - i. ☐ DELETION OF INVENTOR(S)  
Signed statement attached deleting  
inventor(s) named in the prior application,  
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference (useable if Box 4b is checked)  
The entire disclosure of the prior application, from which a copy  
of the oath or declaration is supplied under Box 4b, is considered  
as being part of the disclosure of the accompanying application  
and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission  
(if applicable, all necessary)
- a. ☐ Computer Readable Copy
  - b. ☐ Paper Copy (identical to computer copy)
  - c. ☐ Statement verifying identity of above copies

## ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & documents)
9. ☐ 37 C.F.R. 3.73(b) Statement ☐ Power of Attorney  
(when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503) (2)  
(Should be specifically itemized)
14. ☐ Small Entity Statement(s) ☐ Statement filed in prior application,  
Status still proper and desired
15. ☐ Certified Copy of Priority Document(s)  
(if foreign priority is claimed)
16. ☐ Other:.....

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: \_\_\_\_\_ / \_\_\_\_\_

Prior application information: Examiner: \_\_\_\_\_ Group Art Unit: \_\_\_\_\_

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Attorney's Docket No.: WHIFG98-16pA (dup)

## PRE-SELECTION AND ISOLATION OF SINGLE NUCLEOTIDE POLYMORPHISMS

### RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No.  
5 60/102,069, filed September 28, 1998, the entire teachings of which are incorporated  
herein by reference.

### BACKGROUND OF THE INVENTION

It is becoming clear that human susceptibility to disease and response to  
treatment is influenced by DNA sequence variations. Prominent examples include the  
10 role of variation in ApoE in Alzheimer's disease, CKR5 in susceptibility to infection by  
HIV, Factor V in risk of deep venous thrombosis, MTHFR in cardiovascular disease  
and neural tube defects, various cytochrome p450s in drug metabolism, and HLA in  
autoimmune disease.

Single nucleotide polymorphisms (SNPs) are nucleotide positions at which two  
15 alternative bases occur at appreciable frequency (>1%) in the human population, and are  
the most common type of human genetic variation. These polymorphisms are emerging  
as a critical tool for human genetics in general and pharmacogenomics in particular.  
There is growing recognition that large collections of mapped SNPs provide a powerful  
tool for human genetic studies. A comprehensive collection of SNPs can be used to  
20 identify human disease susceptibility, either directly via association studies (which test  
for enrichment of a specific allele in susceptible individuals) or indirectly via linkage

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disequilibrium studies (which identify the presence of a common ancestral chromosome among susceptible individuals). Because this type of variation is at the sequence level, it also opens a window to the root causes of variation, including differences in gross morphology and biochemistry, and susceptibility to genetic diseases. SNPs can also be  
5 used to create more markers for genetic maps, or to study linkage disequilibrium or human evolution and migration.

Before SNPs can be systematically applied in such studies, however, it is necessary to create a large collection of such loci, construct maps of their genomic locations, and develop methods for large-scale genotyping. The sheer size and  
10 complexity of the genome makes isolation of SNPs cumbersome. In addition, as more polymorphisms are isolated and characterized, there exists the increasing possibility that "new" polymorphisms will be found to be identical to previously-characterized polymorphisms. Furthermore, although there is tremendous variation in the human population, the common SNPs that likely underlie common disease constitute a finite  
15 collection of perhaps 3-6 million total variants.

A variety of approaches can be used to identify SNPs, depending on the desired locus type (*i.e.*, targeted vs. random) and allele frequency (*i.e.*, very common vs. less common). The most direct approach is the targeted resequencing of specific loci; that is, developing a PCR assay for a specific locus, reamplifying the locus from multiple  
20 samples (consisting of individuals and/or pools) and resequencing the resulting products to identify variant bases. Such resequencing can be performed, for example, by using conventional DNA sequencing. Targeted resequencing of specific loci has the advantage that it allows one to study a single locus across many chromosomes. However, targeted resequencing of specific loci has significant disadvantages. It is  
25 expensive and requires interpretation of sequence data from heterozygous samples, which is typically more problematic than that from single alleles.

Another approach is to use known sequence from a database, such as that from the Human Genome Project. Once a sequence of the human genome is known to high accuracy, SNPs can be isolated easily. One would only need to sequence a random

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fragment of human DNA and compare it to the corresponding human reference sequence. The map position of the fragment will be instantly known and every base that differs from the reference sequence will define a SNP. The advantage of the method is that it is technically straightforward and can be carried out at any scale. The  
5 disadvantage is that it requires the availability of a highly accurate reference sequence.

In advance of a complete human genome sequence, one can perform a whole-genome shotgun sequence of multiple individuals. If one obtains sufficient coverage, a given fragment will occur multiple times, allowing one to detect SNPs within that fragment. Weber and Myers (*Genome Res.* 7:401-409 (1997)) proposed shotgun  
10 sequencing to 10X depth from a mixture of individuals as a method to sequence the human genome and to simultaneously identify SNPs. The disadvantage of this approach is that it requires a commitment to sequence the entire genome to several-fold coverage.

Thus, it remains important to develop SNP discovery methods which sequence  
15 the same locus in multiple individuals, maximize sensitivity and specificity, and minimize labor and cost.

#### SUMMARY OF THE INVENTION

The present invention relates to a method of determining or identifying a limited population (a collection) of polymorphisms in a reproducible set of nucleic acid  
20 molecules from one or more nucleic acid-containing samples by analyzing a subset of the nucleic acid molecules. The method described herein does not require PCR and does not require *a priori* knowledge of the sequence of the nucleic acid molecule to be assessed. By limiting the number of polymorphisms under examination to a portion of the total number of polymorphisms that exist in the genome, the method overcomes  
25 many of the disadvantages inherent in identifying SNPs using whole genome sequencing approaches. Furthermore, the method allows sequence comparison of substantially the same subset of nucleic acid molecules across various nucleic acid-containing samples, because each sample will yield substantially the same limited

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population of nucleic acid molecule fragments, i.e., a reduced representation, if treated identically. That is, if a first and second nucleic acid-containing sample are subjected to a particular set of conditions (e.g., digestion with the same restriction endonuclease, such as *Bgl*III, subsequent size separation on an agarose gel, and selection of a particular gel band), each sample will produce substantially the same subset of nucleic acid molecules. This subset of nucleic acid molecules can then be assessed for the presence of polymorphisms (e.g., single nucleotide polymorphisms), with the advantage that each nucleic acid molecule is relatively small in comparison to the untreated nucleic acid molecule in the nucleic acid sample, i.e., is a portion of the original, untreated molecule.

10 In one embodiment, the invention relates to a method for determining or identifying a limited population (or collection) of polymorphisms from nucleic acid molecules in a sample by analyzing a subset of the nucleic acid molecules, comprising the steps of obtaining a nucleic acid-containing sample to be assessed; treating the nucleic acid molecules in said sample to produce nucleic acid fragments selected in a sequence-dependent manner (i.e., a reduced representation) by a method comprising 15 fractionating said nucleic acid molecules to produce nucleic acid fragments, and selecting a subset of said nucleic acid fragments; identifying from said reduced representation subset pairs of nucleic acid fragments corresponding to the same chromosomal locus or location, wherein fragments corresponding to the same chromosomal location are orthologous sequences, and comparing pairs of orthologous 20 sequences to identify polymorphisms between them, thereby determining or identifying a limited population (or collection) of polymorphisms from said nucleic acid-containing sample. In a preferred embodiment, the polymorphisms are single nucleotide polymorphisms.

25 In one embodiment, the nucleic acid molecule is DNA. In another embodiment the nucleic acid molecule is RNA. In a preferred embodiment of the invention, each nucleic acid-containing sample is pooled from more than one individual. For example, the nucleic acid-containing sample can be pooled from individuals who share a

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particular trait (e.g., an undesirable trait, such as a particular disorder, or a desirable trait, such as resistance to a particular disorder).

In a preferred embodiment, the step of fractionating the nucleic acid molecules to produce nucleic acid fragments is performed by one or more restriction  
5 endonucleases (e.g., *Bgl*III, *Xho*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, and *Hae*III). In a preferred embodiment, the step of selecting a subset of said nucleic acid fragments is performed by separating the nucleic acid fragments on an agarose gel and selecting a particular band on the gel. Alternatively, this step can be performed using, for example, high pressure liquid chromatography (HPLC), or by selecting nucleic acid fragments  
10 that hybridize to selected additional nucleic acid sequences.

In one embodiment, the steps of analyzing the reduced representation and/or comparing pairs of orthologous sequences is performed by determining at least a portion of the nucleic acid sequence of the nucleic acid fragments.

The invention also relates to a method for genotyping a nucleic acid-containing  
15 sample from an individual for polymorphisms, the method comprising obtaining a first nucleic acid-containing sample to be assessed; treating said nucleic acid-containing sample to produce a reduced representation of nucleic acid fragments selected in a sequence-dependent manner by a method comprising fractionating said nucleic acid samples to produce nucleic acid fragments and selecting a subset of said nucleic acid  
20 fragments; analyzing the reduced representation to identify pairs of fragments corresponding to the same chromosomal location, wherein fragments corresponding to the same chromosomal location are orthologous sequences; comparing pairs of orthologous sequences to identify polymorphisms therein; obtaining a second nucleic acid-containing sample from an individual to be assessed; and analyzing said second  
25 nucleic acid-containing sample to assess the genotype at one or more of said polymorphisms.

The invention further relates to a method for genotyping a nucleic acid sample for polymorphisms in nucleic acid fragments contained in a reduced representation, comprising the steps of obtaining a nucleic acid-containing sample; treating the nucleic

acid molecules in said sample to produce a reduced representation of nucleic acid fragments selected in a sequence-dependent manner by a method comprising fractionating said nucleic acid molecules to produce nucleic acid fragments and selecting a subset of said nucleic acid fragments; and analyzing the nucleic acid fragments contained in the reduced representation to assess the genotype at one or more polymorphic sites.

In a preferred embodiment, a specific set of criteria is used to determine whether two or more nucleic acid fragments are derived from the same chromosomal location (i.e., whether the fragments are a pair). For example, the criteria can comprise the steps of comparing the sequences of the two members of a proposed pair, wherein the two sequences are further analyzed if the two sequences are at least 80% identical over at least 80% of the length of the shorter of the two sequences; aligning the two sequences, wherein the two sequences are further analyzed if the two sequences are identical over 10 or more bases within the first 50 bases or the last 50 bases of the sequences; identifying candidate single nucleotide polymorphisms, wherein the two sequences are further analyzed if the number of candidate single nucleotide polymorphisms does not exceed 1% of the total number of bases in the shorter of the two sequences, thereby producing a candidate match; repeating the described steps for all proposed pairs; and determining the number of candidate matches for the same chromosomal location, wherein said candidate matches are accepted if said number of matches does not exceed expectations. Accepted candidate matches are considered a pair. In a preferred embodiment, expectations are determined according to binomial or Poisson distributions.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the proportion of SNPs identified (y-axis) as a function of the coverage (x-axis). The five curves, from bottom to top, correspond to  $p$  (minor allele frequency) of 10%, 20%, 30%, 40% and 50%. The proportion of SNPs identified

increases with coverage, and more common SNPs are more rapidly detected than less common ones.

Fig. 2 is a graph showing the relative efficiency (in terms of unique SNPs discovered, x-axis) of detecting a SNP having minor allele frequency  $p$  as a function of the fold coverage (x-axis). The five curves, from bottom to top, correspond to  $p$  of 10%, 20%, 30%, 40% and 50%.

Fig. 3 is a graph showing the expected posterior distribution of allele frequency for SNPs discovered by sampling three chromosomes. As shown by the relatively flat distribution, even though there are more rare SNPs than commonly occurring ones, one is more likely to sample the more common SNPs than the rare ones, simply because of their higher rate of occurrence.

Fig. 4 is a graph showing the number of human restriction fragments with sizes in a 200 bp range centered at a given point, for a typical six-cutter restriction enzyme with an average fragment size of 4 kb.

Fig. 5 is a graph showing the size distribution of inserts for the *Bgl*III and the *Hind*III libraries. Size of the inserts in bp (x-axis) is shown as a percentage of all sequence reads (y-axis). For the *Bgl*III library, the central distribution is 570 bp  $\pm$  17 bp, and 82% of the inserts fall within 2 standard deviations of the mean.

Fig. 6 is a graph showing the estimated complexity for libraries made from various fractions of a *Bgl*III digest, based on the length of the fragments examined (x-axis), and the number of sequencing reads done (y-axis).

Fig. 7 is a flow chart illustrating the steps used to process sequencing reads into pairs.

Fig. 8 is a histogram showing the Poisson-expected (black bars) and observed (white bars) percentages of the total number of reads (y-axis) that fall into groups of sizes 1 through 10 (x-axis), for  $k \approx 1.7$ .

Fig. 9 is a histogram showing the expected distribution of allele frequencies based on the percentage of SNPs examined.



## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of determining a limited population or collection of polymorphisms in a reproducible set of nucleic acid molecules from one or more nucleic acid-containing samples by analyzing a subset of the nucleic acid

5 molecules; the method is referred to herein as “reduced representation shotgun” (RRS). By limiting the number of polymorphisms under examination to a portion of the total number of polymorphisms that exist in the genome, the method overcomes many of the disadvantages inherent in identifying SNPs using whole genome sequencing approaches. Furthermore, the method allows sequence comparison of substantially the

10 same subset of nucleic acid molecules across various nucleic acid-containing samples, because each sample will yield substantially the same limited population of nucleic acid molecule fragments if treated identically. That is, if a first and second nucleic acid-containing sample are subjected to a particular set of conditions (e.g., digestion with the same restriction endonuclease, such as *Bgl*III, subsequent size separation on an agarose

15 gel, and selection of a particular gel band), each sample will produce substantially the same subset of nucleic acid molecules. This subset of nucleic acid molecules can then be assessed for the presence of polymorphisms (e.g., single nucleotide polymorphisms), with the advantage that each nucleic acid molecule is relatively small in comparison to the untreated nucleic acid molecule in the nucleic acid sample, i.e., is a portion of the

20 original, untreated molecule.

By “a limited population of polymorphisms” and “a collection of polymorphisms” is meant a subset of the total polymorphic loci potentially available within the nucleic acid sample. If the nucleic acid sample is total genomic DNA, for example, then a “limited population of polymorphisms” is a population of

25 polymorphisms that represents a subset of the total number of polymorphisms present in the entire genome of the organism.

As used herein, “substantially the same” is intended to mean at least 70%, preferably 80%, more preferably 90%, and most preferably 95% (or more) identity. However, one of ordinary skill in the art will recognize that there are situations in which

complete concordance between limited populations of polymorphic is not possible. For instance, when polymorphisms are isolated from the first nucleic acid fraction, and then assayed in the equivalent fraction from another individual (*i.e.*, a nucleic acid fraction created by the same techniques as those used to produce the nucleic acid fraction from which the limited population of polymorphisms was first isolated), the loci found in the two fractions will differ slightly to the extent that polymorphisms exist which alter the underlying and, in general, constant property of the sample upon which the fractionation and/or separation is based, for example, the restriction fragment site or length. For instance, DNA from two individuals cut with *EcoRI* will differ if there is a nucleotide difference within an *EcoRI* site. Put another way, the very differences that are seen in RFLP studies will also be seen in practicing the present invention, if restriction enzymes are used to create the nucleic acid fractions. However, the frequency of such RFLPs is generally relatively low (estimated to be less than 1% of such fragments) and so this does not pose a significant problem; non-restriction endonuclease-based methods can be used in these instances.

Accordingly, the method of the invention comprises the steps of obtaining a nucleic acid-containing sample to be assessed; treating nucleic acid molecules in said sample to produce nucleic acid fragments selected in a sequence-dependent manner by a method comprising fractionating said nucleic acid molecules to produce nucleic acid fragments, and selecting a subset of said nucleic acid fragments, thereby producing a reduced representation; analyzing the reduced representation to identify pairs of fragments corresponding to the same chromosomal location, wherein fragments corresponding to the same chromosomal location are orthologous sequences; and comparing pairs of orthologous sequences to identify polymorphisms therein.

As used herein, a nucleic acid-containing sample (also referred to as nucleic acid sample or sample) is intended to include any source or sample which contains nucleic acid (*e.g.*, which contains nucleic acid molecules such as RNA or DNA). The sample can be, for example, any nucleic acid-containing biological material (including, but not limited to, blood, saliva, hair, skin, semen, biopsy samples, and one or more cells). The

sample can be obtained from any organism, including bacteria, viruses, plants, insects, reptiles and mammals (e.g., humans). The sample can contain nucleic acid from one or more individuals or organisms; that is, the sample can be from a single individual or organism or can be a pooled sample from multiple individuals or organisms.

- 5 For example, it may be desirable to pool samples from individuals or organisms who share a particular trait. The trait may be a desirable trait (e.g., an increase in a desirable attribute such as intelligence, resistance to a particular disorder or resistance to infection by a particular organism, or a decrease in an undesirable attribute such as a reduced incidence of a particular disorder), or an undesirable trait (e.g., an increase in an undesirable attribute or a decrease in a desirable attribute). Alternatively, it may be desirable to pool samples from individuals sharing a familial relationship. Nucleic acid samples can also be obtained from defunct or extinct organisms, e.g., samples can be taken from pressed plants in herbarium collections, or from pelts, taxidermy displays, fossils, or other materials in museum collections. The sample can also be a sample of isolated nucleic acid molecules, e.g., isolated DNA or DNA contained in a vector.
- 15 Suitable nucleic acid samples also include essentially pure nucleic acid molecules, nucleic acid molecules produced by chemical synthesis, by combinations of biological and chemical methods, and recombinantly produced nucleic acid molecules (see e.g., Daugherty, B.L. *et al.* (1991) *Nucleic Acids Res.* 19(9):2471-2476; Lewis, A.P. and Crowe, J.S. (1991) *Gene* 101:297-302).
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As used herein, "nucleic acid molecule" is intended to include, but is not limited to, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), cDNA, nucleic acids from mammals or other animals, plants, insects, bacteria, viruses, or other organisms.

- According to the method, the nucleic acid-containing sample is treated to produce a subset or reduced representation of nucleic acid fragments selected in a sequence-dependent manner. For example, the sample can be subjected to fractionation and selection methods which, when combined, are sequence-dependent, and produce a subset of nucleic acid molecules from the original sample. Either or both of the fractionation and selection steps can be sequence-dependent. "Sequence-dependent
- 25

manner” is intended to mean that the method relies on the underlying nucleic acid sequence in accomplishing its purpose.

For example, the nucleic acid sample can be fractionated (*e.g.*, in a random or sequence-dependent manner), then subjected to a selection step that is sequence-dependent (*e.g.*,  
5 based on methylation patterns), or the nucleic acid sample can be fractionated in a sequence-dependent manner (*e.g.*, with restriction endonucleases), and then a subset can be selected (*e.g.*, with agarose gels or HPLC), or both the fractionation and selection steps can be sequence-dependent.

As used herein, “fractionating the nucleic acid molecules” is intended to include  
10 methods which produce fragments of the nucleic acid molecules in the original sample. These fragments are generally smaller (*i.e.*, comprise fewer nucleotides) than the nucleic acid molecules in the original nucleic acid sample. This step can be performed by biochemical, mechanical or physical means. For example, suitable methods include, but are not limited to, cleavage with restriction endonucleases, shearing, exposure to  
15 ultraviolet light and exposure to radiation. Additional methods include, for example, techniques that target introns, exons, signal sequences, methylation, glycosylation patterns, recognition sites for DNA binding proteins, etc. For example, a nucleic acid sample can be fractionated via treatment with one or more restriction endonucleases (*e.g.*, *Bgl*II, *Xho*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Hae*III) to produce nucleic acid  
20 fragments. Preferably the selected restriction endonuclease(s) cleave the nucleic acid molecule at approximately every 2000 bases.

Examples of fractionating nucleic acid samples in a sequence-dependent manner include methods which cleave or break nucleic acid molecules in a way that is repeatable with respect to the nucleic acid sequence. Cleavage by means of one or more  
25 restriction endonucleases is a preferred example of such sequence-dependent cleavage; for example, a given restriction enzyme reliably cuts nucleic acid at a specified sequence, *e.g.*, *Eco*RI cuts at the sequence “G|AATTC”. Sequence-dependent fractionation methods which do not specifically utilize restriction endonucleases may also be useful. For example, a method that reliably cleaved nucleic acid in the vicinity

of methylated regions would tend to be "sequence-dependent" because methylation patterns tend to be conserved. In addition, some proteins, such as ribozymes, can be designed to cleave nucleic acid at a desired site. Chemicals, ultraviolet light, radiation and other methods can also be used to effect the sequence-dependent fractionation if

5 they can be made to cleave the nucleic acid at similar chromosomal positions between different nucleic acid samples. If the fractionation step is not sequence-dependent, then the selection step should be sequence-dependent.

Suitable methods for selecting subsets of the fractionated nucleic acid molecules include, but are not limited to, size separation such as separation on an agarose gel or

10 via high pressure liquid chromatography (HPLC). A subset of the total fragments can then be selected by cutting out a portion of the gel and isolating the nucleic acid fragments within the cut-out portion of the gel. The selected nucleic acid fraction can be in a broad or narrow size range, *e.g.*, 10 bases to 1000 bases, or more. More preferably, the selected fraction is from about 300 base pairs to about 1000 base pairs,

15 such as from about 380 base pairs to about 480 base pairs, from about 400 base pairs to about 500 base pairs, from about 480 base pairs to about 580 base pairs, from about 500 base pairs to about 600 base pairs, from about 540 base pairs to about 640 base pairs, from about 380 to about 640 base pairs, from about 380 to about 500 base pairs, or from about 400 to about 600 base pairs. Selection of the subset of nucleic acid fragments

20 can also be performed in a sequence-dependent manner. For instance, mechanical shearing of nucleic acid molecules generally breaks up nucleic acid at random intervals. However, mechanical shearing, followed by selection of those fragments that contain, *e.g.*, exon-specific sequences, produces a nucleic acid fraction the composition of which is dependent on the underlying nucleic acid sequence. Additionally, nucleic acid

25 fragments can be selected by hybridization to a selected set of nucleic acid molecules (*e.g.*, probes).

This subset of nucleic acid fragments selected in a sequence-dependent manner (*i.e.*, a reduced representation) is analyzed to identify pairs of nucleic acid fragments corresponding to the same chromosomal locus or location. That is, a fragment from a

particular chromosomal location is paired with one or more other fragments which are from the same chromosomal location. The fragments which are paired can be two alleles from the same individual, or two or more alleles from different individuals. The analysis can be performed, for example, by sequencing at least a portion of the nucleic acid fragments. Fragments corresponding to the same chromosomal location are termed "orthologous sequences".

In one embodiment of the invention, specific criteria are used to determine whether two or more fragments form a pair of orthologous sequences. These criteria are designed to exclude, i.e., not include as pairs, fragments which do not occur at the same chromosomal location. For example, sequences to be excluded include highly homologous sequences, or duplicated loci (repeats), which occur at different chromosomal locations.

In one embodiment, every fragment is compared against all other fragments using analysis steps comprising: (a) comparing the sequences of the two members of a proposed pair, where the two sequences are further analyzed if the two sequences are at least 80% identical over at least 80% of the length of the shorter of the two sequences, (b) aligning the two sequences identified from (a), where the two sequences are further analyzed if the two sequences are identical over 10 or more bases within the first 50 bases and the last 50 bases of the sequences, (c) identifying candidate single nucleotide polymorphisms in the sequences of (b), where the two sequences are further analyzed if the number of candidate polymorphisms does not exceed 1% of the total number of bases in the shorter of the two sequences, where two sequences which meet the criteria of (a) - (c) qualify as a candidate match, (d) repeating (a) - (c) for all proposed pairs, and (e) determining the number of candidate matches for a given chromosomal locus, where the candidate matches are accepted if the number of matches does not exceed expectations. In this method, the expectations can be determined, e.g., according to binomial or Poisson distributions. Two fragments that meet all of the above criteria are considered a pair.

Fragments of a pair are then compared to identify polymorphisms, e.g., by determining at least a portion of the nucleic acid sequence of the fragments. As used herein, a polymorphism is an allelic variation between two samples. As used herein, the term preferably refers to single nucleotide polymorphisms (SNPs), but can also include differences in proteins (e.g., isozymes, blood groups, blood proteins), differences in nucleotide sequence (e.g., restriction site maps), or differences in length of a stretch of nucleic acid (e.g., RFLPs (restriction fragment length polymorphisms), microsatellites, STRs (short tandem repeats), SSRs (simple sequence repeats), SSLPs (simple sequence length polymorphisms), and VNTRs (variable number tandem repeats)). A polymorphism is not limited by the function or effect it may have on the organism as a whole, and can therefore include allelic differences which may also be a mutation, insertion, deletion, point mutation, or structural difference, as well as a strand break or chemical modification that results in an allelic variant. A polymorphism between two nucleic acids can occur naturally, or be caused intentionally by treatment (e.g., with chemicals or enzymes), or can be caused by circumstances normally associated with damage to nucleic acids (e.g., exposure to ultraviolet radiation, mutagens or carcinogens).

A "single nucleotide polymorphism," or SNP, is a difference of a single base between two homologous nucleic acids. For example, a diploid mammal having the sequence "GCTTCCG" at a particular position on one copy of chromosome 12, and the sequence "GCTACCG" at the same position on the other copy of chromosome 12, exhibits a SNP at that position, and is heterozygous for that SNP. If the individual were homozygous (e.g., had two copies of the sequence "GCTTCCG"), that SNP would not be visible within a sample of that individual's DNA, but the SNP would be visible when compared to the DNA of an individual that were either heterozygous for that SNP (e.g., had the alleles "GCTTCCG" and "GCTACCG"), or were homozygous for a different allele of that SNP (e.g., "GCTACCG"). The genotype of a SNP in a sample is generally accomplished by sequencing, e.g., with an M13 vector.

By "determining polymorphisms" is meant that the polymorphic loci within the nucleic acid are assayed, and the differences determined between the polymorphic locus in one nucleic acid and the polymorphic locus in another nucleic acid.

It will be understood that any of the steps of the methods described herein can be carried out physically or virtually. That is, for example, nucleic acid molecules can be physically subjected to treatment with one or more restriction enzymes, or the sequence of the nucleic acid molecule can be analyzed virtually, e.g., with computer software, to identify restriction sites for one or more restriction enzymes, and the resulting cleaved nucleic acid fragments can be shown virtually. As used herein, "virtually" is intended to mean without physical or actual manipulation.

For example, one way of reproducibly determining the same limited population of polymorphisms across different nucleic acid samples would be as follows: (1) nucleic acid samples from several individuals are isolated and pooled; (2) the pooled nucleic acid sample is then fractionated in a sequence-dependent manner, e.g., cut with one or more restriction enzymes; (3) the fractionated nucleic acid sample is then separated by size; (4) a size fraction is selected; (5) pair of sequences from the same chromosomal locus are selected; and (6) polymorphisms are isolated from that fraction. Other nucleic acid samples that are to be tested are then treated in the same manner, and then assayed for those same polymorphisms. To identify more polymorphisms from the original sample, the process can be repeated using a different size fraction. This approach greatly reduces the possibility of re-isolation of previously-identified polymorphisms. Alternatively, instead of using a different size fraction as the source of new polymorphisms, pooled nucleic acid can be collected from individuals unrelated to the individuals previously used. Alternatively, one or more different fractionation methods may be used.

One application of the present invention comprises (i) combining total genomic DNA from multiple individuals; (ii) digesting the mixture with a restriction enzyme (e.g., *HindIII*); (iii) subjecting the resulting DNA to electrophoresis on a gel; and (iv) excising a particular band which represents or includes fragments of a particular size



and cloning the restriction fragments within a specific size range (*e.g.*, 500-600 bp). Such a library represents a specific subset of the genome, containing essentially the same fragments from each individual. Within this specific subset, fragments from a particular chromosomal locus are paired to facilitate comparison of nucleic acid  
 5 sequences from several individuals at that locus. These pairs are then assayed for the polymorphic loci contained therein.

In the present invention, any nucleic acid-containing sample can be directly compared to any other nucleic acid sample by simply treating the second sample in the same way as the first, *e.g.*, by digesting with *HindIII*, electrophoresis on an agarose gel,  
 10 and selection of the 500-600 bp fraction. The resulting nucleic acid fraction will contain substantially the same polymorphic loci as the nucleic acid fraction from the first nucleic acid sample. Nucleic acid samples from different individuals, or from different pools of individuals, if all treated similarly, will generally produce substantially similar subsets of nucleic acid fragments, and therefore similar subsets of  
 15 polymorphic loci within those subsets of nucleic acid fragments.

Many uses of SNPs require: (i) the SNP's map position in the human genome, and (ii) a genotyping assay for scoring the locus in association studies. Even if the SNPs are mapped, they cannot be used without a genotyping assay. The reduced representation approach has a powerful feature that may facilitate efficient genotyping.  
 20 If one wishes to genotype a new sample for 10,000 SNPs isolated from a specific size fraction (*e.g.*, *HindIII*/500-700 bp), one could restriction-digest the sample; ligate a generic linker; isolate the appropriate size fraction; and amplify by PCR using primers complementary to the generic linker. The resulting amplification products could be hybridized to an appropriate 'genotyping array'. It is known that (i) such amplicons  
 25 provide a sample with significantly reduced complexity (Lisitsyn *et al.* (1993) *Science* 259:946-51) and (ii) samples with such reduced complexity can be used as efficient probes for hybridization to DNA arrays (as shown by hybridization of mRNA to expression monitoring arrays (Lockhart, D.J. *et al.* (1996) *Nature Biotech.* 14:1675-

1680). This approach has the advantage that it does not require developing specific PCR assays for each of 10,000 loci.

If additional polymorphisms are required, they can be isolated from a new fraction, which is selected to differ from the previous fraction. The new fraction can  
 5 differ from the previous in the technique used to fractionate the nucleic acid, the method used to select the nucleic acid fragments, or a new subset of nucleic acid fragments can be selected, *e.g.*, if the 500-600 bp *HindIII* fraction were chosen previously, then the 600-900 bp fraction can now be chosen, or a 500-600 bp *PstI* fraction can be used. The distribution of restriction enzyme sites is roughly uniform across the genome, with the  
 10 exception of sites containing the CpG dinucleotide, and the size of restriction fragments therefore follows an exponential distribution. For a restriction enzyme with average fragment size  $d$ , digesting a genome of size  $G$ , the number of unique fragments ( $D$ ) in the size range  $[x_1, x_2]$  is estimated by:

$$D = (G/d)(e^{-x_1/d} - e^{-x_2/d})$$

15 For a typical six-cutter enzyme, the average fragment size ( $d$ ) is 4 kb, and thus  $D$  [400, 600] is 33,000. This represents 16 Mb, or 0.5% of the human genome. This model presumes that all fragments in the size range are equally represented, and laboratory techniques for selecting fragments based on size may result in a skewed distribution. Further guidance for the practitioner is provided in the examples.

20 The invention also provides for a method for making a genotyping chip for use in assaying a limited population of polymorphisms within a sample (see, *e.g.*, U.S. Pat. Nos. 5,861,242 and 5,837,832). Once a set of polymorphisms is isolated, probes or primers for detecting those polymorphisms can be incorporated into such a chip. When it is desirable to assay an individual for the polymorphisms in the set, nucleic acid is  
 25 isolated from that individual, and it can be fractionated with the same methods that were used to isolate the original set of polymorphisms. For example, if nucleic acid from 10 individuals can be pooled, cut with *EcoRI*, and the polymorphisms isolated from the 2000 bp fraction, and primers or probes for detecting those polymorphisms can be placed on a genotyping chip. The nucleic acid from an individual to be tested could

also be restricted with *EcoRI*, and the 2000 bp fraction isolated, ligated to a generic primer, and amplified based upon that primer, and applied to the genotyping chip. The method of the invention therefore allows the user to concentrate study on only a limited portion of the entire spectrum of the available polymorphisms. By examining only a

5 limited portion of the genome, this method has the added benefit of reducing cross-reactivity between unrelated genetic sites.

The methods of the present invention can be used in humans and non-humans. For example, the methods can be used to assay polymorphisms in animals for veterinary purposes. For instance, they can be used to amplify target sequences known to be

10 associated with susceptibilities to diseases with genetic components, or to detect known genetic defects in purebred animals such as dogs or horses. They can also be used to assess levels of biodiversity in populations of animals, plants, or microorganisms. The can be applied in the search for beneficial genetic components in animals and plants, both domesticated and wild, that are used for food, feed, fiber, oils, lumber, or other raw

15 materials. They can be applied in the search for genetic components of strains of pests, parasites or disease organisms that are especially virulent to humans, plants or animals.

The methods of the invention can also be used to amplify sequences across species. For instance, chimpanzees and humans share approximately 99% sequence similarity. The methods of the invention can be used to locate those areas in which the

20 1% interspecific difference is located, thereby pinpointing the "evolutionary hotspots" responsible for species differentiation, and interspecific conserved regions, as well.

The invention also relates to a method for genotyping a nucleic acid sample for polymorphisms in nucleic acid fragments contained in a reduced representation, comprising the steps of obtaining a nucleic acid-containing sample; treating the nucleic

25 acid molecules in said sample to produce a reduced representation of nucleic acid fragments selected in a sequence-dependent manner by a method comprising fractionating said nucleic acid molecules to produce nucleic acid fragments and selecting a subset of said nucleic acid fragments; and analyzing the nucleic acid fragments contained in the reduced representation to assess the genotype at one or more

polymorphic sites. For example, the step of analyzing can be performed by attaching specific oligonucleotide linker sequences to the fragments in the reduced representation and then amplifying said fragments, such as by polymerase chain reaction using primers complementary to the linker sequences. Alternatively, amplification can be performed

5 by methods including, but not limited to, cloning the fragments in an organism, performing single-base extension reactions on the reduced representation, hybridization to oligonucleotide arrays, and oligo ligation assays. In a particular embodiment, the sample is genotyped for polymorphisms identified by reduced representation methods described herein. In a preferred embodiment, the sample from the individual to be

10 assessed is treated to produce a reduced representation with a method identical to that used to identify the polymorphisms which are to be genotyped.

The methods of the invention can also be selected and used to fingerprint proprietary biological material. For example, a set of polymorphisms can be chosen corresponding to specific genotypes known to exist in a protected crop cultivar. Assays

15 of plants can be made according to the present invention, to determine if those plants correspond to the genotype of the patented cultivar.

The invention will be further illustrated by the following non-limiting examples. The teachings of all references cited herein are incorporated herein by reference in their entirety.

## 20 EXAMPLES

Example 1: Theoretical basis of SNP sampling.

A. Identifying SNPs by Poisson sampling. If a reduced representation library from a mixture of many individuals is sequenced to k-fold coverage, the probability of identifying a SNP with minor allele frequency  $p$  is:

$$25 \quad \sum_{i=1}^{\infty} \pi(i,k) [1 - p^i - (1-p)^i]$$

where  $\pi(i,k)$  is the Poisson probability that the fragment containing the SNP is sampled  $i$  times and the bracketed term is the probability that both alleles occur in the sample.

As shown in Fig. 1, the proportion of SNPs increases with coverage and more common SNPs are more rapidly detected than less common ones. Fig. 1 also shows that there are diminishing returns to deep sampling. Beyond a certain point, each additional 1x coverage yields fewer SNPs. Rather than sampling more deeply, it is more  
 5 advantageous to begin sampling of a new library (*i.e.*, a new nucleic acid fraction).

The optimal sampling depth can be determined by calculating the “efficiency”, *i.e.*, the proportion of SNPs found divided by the coverage. Fig. 2 shows the relative efficiency (*i.e.*, new SNPs per read). Strikingly, the efficiency is maximized at around 2.5-fold coverage for SNPs with minor allele >20%—although the peak is relatively  
 10 broad.

B. Distribution of allele frequencies. It is desirable to identify SNPs that are reasonably polymorphic in the general population, and the distribution of allele frequencies of SNPs identified in a reduced representation approach can be predicted from population genetics theory. These predictions can be compared to observed data. According to  
 15 population genetics theory (Nei, M. (1987) *Molecular Evolutionary Genetics*, Columbia University Press, New York), the distribution of allele frequencies for all polymorphisms in a population follows the equation

$$F(p) = C [p(1-p)]^{\theta-1},$$

where  $C$  is a constant of proportionality and  $\theta$  is the classical parameter  $4N\mu$  (estimated  
 20 by  $\pi$ , below). For the human population, Wang *et al.* ((1998) *Science* 280:1077-1082) have estimated  $\theta$  to be approximately 0.0004.

Rare alleles are less likely to be observed in a small sample. The allele frequency distribution for variants observed in a sample of  $i$  chromosomes can be determined by Bayes' theorem, using the weighting factor  $[1 - p^i - (1-p)^i]$ , which reflects  
 25 the chance that any given SNP will be encountered during sampling of  $i$  chromosomes. For SNPs found in a sample of three chromosomes, the allele frequency distribution is shown in Fig. 3, which shows that the allele frequency distribution of SNPs discovered in a small sample of chromosomes is expected to be quite flat. That is, the allele

frequency of SNPs identified from a small sample is expected to be roughly uniformly distributed in the range [0,1]. The mean frequency of the minor allele is expected to be just under 25%, corresponding to heterozygosity of about 35%. These theoretical expectations agree reasonably well with the empirical finding of Wang *et al.* ((1998) 5 *Science* 280:1077-1082). It also follows from this distribution that the maximal efficiency for identifying common (> 20%) SNPs is expected at 2-4-fold coverage. Thus, those SNPs found in a small sample are suitably biased toward having a reasonable allele frequency in the population.

C. Number of fragments in a size range. The distribution of restriction sites tends to be 10 uniform across the human genome (with the exception of restriction sites containing the CpG dinucleotide) and thus the size of restriction fragments follows an exponential distribution. For a restriction enzyme with average fragment size  $d$ , the number of restriction fragments in the size range  $[x_1, x_2]$  is:

$$(G/d) (e^{-x_1/d} - e^{-x_2/d}),$$

15 where  $G$  is the genome size. For a typical six-cutter with an average fragment size ( $d$ ) of about 4 kb, the number of fragments in a size window of 200 bp is shown in Fig. 4.

D. Implications. There are roughly 33,000 fragments in the range or 400 bp - 600 bp. Because such fragments could be sequenced in a single pass, it would require about 33,000k successful sequencing reads to obtain k-fold coverage. There are roughly 20 22,000 fragments in the range 1.9 kb - 2.1 kb. Because each fragment contains two distinct ends (of which only one is seen in a single sequencing read), there are a total of 44,000 distinct ends, and it would require about 44,000 k successful sequencing reads to obtain k-fold coverage. Reduced representation libraries are therefore of an appropriate size for discovery of SNPs. For example, obtaining 4-fold coverage would require in the 25 range of 150,000 successful sequence reads and would survey roughly 20 Mb of genomic DNA.

E. Monitoring a library by resampling. It is not necessary to wait until 150,000 sequences have been obtained in order to test whether a reduced representation project is proceeding successfully. It is possible to monitor the success of the project by monitoring the resampling rate, *i.e.*, the frequency at which fragments are seen multiple  
5 times.

If one performs  $N$  successful sequence reads from a library with  $D$  distinct sequences (where  $D$  is the complexity, and is either (1) the number of fragments if the fragments are small enough to be fully sequenced in a single read or (2) the number of ends if the fragments are too large to sequence in a single read), then the number of  
10 pairwise matches is  $N^2/2D$ . Each match will contain SNPs at a rate determined by the nucleotide diversity,  $\pi$ , which is defined as the per nucleotide pairwise difference between two chromosomes drawn from a population. Large-scale surveys of random DNA estimate  $\pi$  at  $4 \times 10^{-4}$ , or 1 difference per 1200-2500 bp. Thus, in a reduced representation library containing 400-600 bp fragments, approximately 1 in 4 paired  
15 sequences should contain a SNP. It follows from the low rate of true SNPs ( $5 \times 10^{-4}$ ) that false positives can be avoided with 95% accuracy, only if incorrect basecalls are exceedingly rare ( $< 2.5 \times 10^{-5}$ ).

Thus, digestion of the human genome with a six-cutter restriction endonuclease, followed by size selection of 400-600 bp fragments, should result in a library containing  
20 a complexity of 30,000 - 40,000 unique genomic loci. If the library is oversampled such that individual loci are seen more than once, SNPs should be found in one out of four paired reads. If the average number of chromosomes sampled is low, the average allele frequency of the resulting variants should be biased towards highly heterozygous SNPs.

#### Example 2. Sample Reduced Representation Strategy

25 To prepare reduced representation libraries, DNA is isolated from 10-20 individuals. These are then combined in equimolar amounts to create pooled DNA. A collection of reduced representation libraries is then prepared by digesting the DNA with a standard six-cutter enzyme (such as *HindIII*); size-fractionating it by gel

electrophoresis and/or preparative HPLC; and creating a series of libraries, with each representing a distinct fraction and containing 30,000-40,000 distinct sequences.

SNPs are then identified by sequencing each library to 4.5-fold coverage. Theory suggests that the optimal depth is about 3x, although the optimum is relatively broad.

- 5 Slightly deeper coverage may be appropriate to allow for imperfect fractionation. Yield should be monitored and adjusted accordingly.

A small proportion of false positives is acceptable, as these will be identified and excluded in the course of developing genotyping assays, but as the accuracy should be as high as possible, candidate SNPs should be confirmed. Past experience indicates that

- 10 SNPs should be able to be identified with greater than 95% accuracy, *i.e.*, >95% of apparent SNPs will be actual SNPs. As a quality assessment measure, a subset of SNPs should be "confirmed" in order to estimate (i) accuracy and (ii) allele frequency. This can be done by testing 100 candidate SNPs by developing PCR assays; amplifying them from ten samples (*e.g.*, 7 individuals and three pools of 50 chromosomes from distinct  
15 ethnic groups), and resequencing the products to confirm the presence and frequency of the SNP.

To calculate the yield of SNPs, one can consider the following example:

	Frequency of useful SNPs found with 2-fold coverage:	1 per 2 kb
	Sequencing read length:	500 bp
20	Sequencing pass rate:	85%

This implies a yield of:

$$\frac{(\text{fold coverage} \times \text{frequency useful SNPs})}{(\text{sequencing read length} \times \text{sequencing pass rate})}$$

or:  $(4.5 \times 2000)/(500 \times 0.85)$ , or 1 SNP per 21.2 sequencing reads.

- 25 In general, there should be one SNP every 1000 bp, but a proportion (~1/3) will be in repetitive sequence that is suboptimal for subsequent genotyping.



### Example 3. Empiric Results.

Two size-selected libraries were constructed from a diverse pool of ten individual humans (4 Caucasian (1 each of :Utah, French, Amish, Russian), 1 each of: Japanese, Chinese, African American, African Pygmy, Melanesian, Amerindian). The pooled DNA was digested to completion with either *Bgl*II or *Hind*III, and fragments were prepared in a narrow range around 500 bp for the *Bgl*II digestion, and around 600 bp for the *Hind*III digestion, using preparative agarose gel electrophoresis. The resulting size fractions were cloned into M13-based vectors, and individual clones were sequenced. The size distributions obtained were appropriately narrow, as is shown in Fig. 5, which is a graph showing the size distribution of inserts for the two libraries. For example, the central distribution of the *Bgl*II library had a mean insert length of 570 bp  $\pm$  17 bp. Only 84% of the sequencing reads fell within two standard deviations of the mean, as a long flat tail of contaminating sequences of various lengths was observed. This is expected, given that the sieving properties of agarose gels are known to be imperfect, with some small fragments traversing the gel more slowly than expected, and some larger fragments moving more quickly than expected.

The complexity of the libraries was next determined, as the goal of reduced representation is to facilitate resampling of individual chromosomal loci. Estimated complexity for the *Bgl*II library is shown in Fig. 6, which shows the estimated complexity for libraries prepared from various size fractions (x-axis) of a *Bgl*II digest, and the number of sequencing reads done (y-axis).

The sequencing reads were then processed as shown in Fig. 7. BLAST was first used to identify reads that were highly similar in sequence to one another, that is, the reads that had greater than 400 bp of identity, but any method of searching on the basis of similarity, and reporting on the extent of sequence similarity between pairs of reads can be used. To accurately measure the rate of resampling and find SNPs, reads must be paired only with truly orthologous sequences. The following criteria were used, after considering the expected polymorphisms between two nucleic acid fragments derived from the same locus. Once every read was compared against every other read, a pair of

- reads were allowed to continue through the process if, over 400 bp or more, there was 80% or more sequence identity over 80% of the length of the shorter of the two reads. Reads passing through this step were then aligned. Several criteria were applied to the aligned sequences. First, because sequence quality is often lower at the ends of reads, a
- 5 10 base pair window was examined within the first and last 50 base pairs. If the two sequences did not match perfectly within the window, the window was repeatedly shifted one base towards the middle of the alignment, and the two sequences within the newly placed window were compared again. If no 10 base pair window matched within the first 50 base pairs (at either end), then the pair was not analyzed further. If there was
- 10 a perfect match in a 10 base pair window within the first 50 bases of both ends, then the pair was analyzed further. This step serves to eliminate sequences with unclear sequence at either end, as well as sequences which are too short relative to each other. That is, there is no separate "trimming" step after alignment, as differences in length between two reads are viewed as a defect. The 10-base window within 50 bases of the
- 15 end to work very effectively, but other sizes of windows can be used over longer distances from the ends if this is required to attain the desired sequence quality. Alternatively, this window and distance can be shortened, or this step may be eliminated altogether, if the sequence quality is deemed high enough to not require such rigorous standards.
- 20 Second, it was determined whether there were any SNPs in the pair of reads. In making this determination, quality of the sequence was also assessed. That is, differences between two reads were not assumed to be SNPs, but rather, the sequence itself was evaluated for quality, to determine if a difference was really a polymorphism, or a difference in basecalling between the two reads.
- 25 Third, since repetitive DNA was present in the libraries, it is necessary to avoid pairing sequences that originate from distinct, if homologous, genomic loci. To accomplish this, the low nucleotide diversity in the human genome ( $\pi = 1/2000$  bp) was considered, and it was concluded that any true match should have considerably less than 1% candidate SNPs. Thus, any candidate pair with > 1% high-quality discrepancies

were eliminated. Specifically, the number of SNPs in an alignment were counted. If the total number of SNPs exceeded 1% of the bases, then the pair was rejected on the assumption that the two reads of the pair represented a duplicated or repetitive locus.

For example, if sequences A, B, C and D are placed in a group as possibly  
 5 representing a single locus, then each would be compared to the other. If the number of SNPs found between A and B make up less than 1% of their length, then A and B continue to be considered as being from the same locus. But if the comparison between C and D shows that SNPs make up 2.% percent of the differences between them, and either C or D, when compared to either A or B, have SNPs making up 1.2% of the  
 10 differences in each comparison, then A and B are concluded to be sequences containing "true" SNPs, while C and D are considered to represent duplicated or repeated loci.

Alternatively, if one wishes to exclude all loci that are related to duplicated or repetitive loci, then the entire group of reads can be excluded.

All such pairs that passed the above steps were collapsed into connected  
 15 component groups, each corresponding to a putative single genomic locus. Such stringent criteria may eliminate a small number of loci that are truly highly diverse, but this was deemed to be outweighed by the concern of inappropriate pairing of non-orthologous sequences. Once paired reads were identified, the rate of matches was examined and compared to that predicted, that is, the reads were assessed for the size of  
 20 their group. For a library sequenced to  $k$ -fold coverage, the probability that exactly  $i$  orthologs of a given read are sequenced is estimated by the Poisson probability,  $\pi(i,k)$ . In this method, given an estimation for the number of sequences amongst the nucleic acid fragments which represent a single locus, and given a certain number of sequences examined, either the binomial or Poisson distributions can be used to determine these  
 25 expectations. The Poisson distribution is shown for the *Bg/III* library in Fig. 8, which is a histogram showing the Poisson-expected (black bars) and observed (white bars) percentages of the total number of reads (y-axis) that fall into groups of sizes 1 though 10 (x-axis), for  $k \approx 1.7$ .

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For example, groups with exactly 4 mutually matching reads (groups of exactly 4 putatively orthologous reads) are together expected to comprise about 5-10% of the total number of reads, while the reads assigned to putatively orthologous groups of size 10 involve only about 1% of all reads. Groups that are large enough that they are  
 5 expected to occur less than once, based on the Poisson distribution, are discarded and non of the potential SNPs occurring between reads of these large groups are accepted.

Initial calculations modeled complexity as  $D$  unique inserts, which were to be represented equally in the library. The observed size distribution was, however, skewed, as expected, due to the known imperfections of agarose gel as a sieve. That is,  
 10 a band cut out of a gel in the range of 500 to 600 base pairs contains fragments the sizes of which produce a bell-shaped curve, with tails extending below 500 bp and above 600 bp. The effective complexity, defined as the chance that any two reads drawn from the library would constitute a match, was then measured, and the results are show in Table 1, below.

- 15 Table 1. Complexity of *Bgl*III and *Hind*III libraries.  
 Complexity = number of reads<sup>2</sup>/(2 x number of pairs), and assumes that all fragments are equally represented in the library.

Library	<i>Bgl</i> III	<i>Hind</i> III
Reads	17,130	4,570
20 Pairs	14,490	502
Complexity	9,839	20,797
Repeat Content	6%	6%

Analysis of large numbers of clones from the *Bgl*III library revealed 14,000 paired reads, demonstrating an effective complexity of 10,000. Similarly, analysis of  
 25 23,000 clones from the *Hind*III library revealed an effective complexity of about 20,000. Furthermore, considering the skewed size distribution of reads, the rate at which reads match one another closely fits theoretical expectation, as is shown in Fig. 8,

which is a histogram showing the Poisson-expected (black bars) and observed (white bars) percentages of the total number of reads (y-axis) that fall into groups of sizes 1 through 10 (x-axis) for  $k \approx 1.7$ .

- 5 The *Bgl*III and *Hind*III libraries were shown to have the desired properties for use in the invention, producing about 1,650 SNPs from 19,000 reads, or about 1 SNP per 11 reads performed. This compares quite favorably with the results of Wang *et al.* (1998) (*Science* 280:1077-1082), in which 1 SNP was found per 12 reads for 3 DNAs screened, and 1 SNP per 48 chip hybridizations when 8 DNAs were screened. The allele frequency of these SNPs was also high, as expected from theory (Fig. 9).
- 10 All references, patents and patent applications are incorporated herein by reference in their entirety. While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

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## CLAIMS

What is claimed is:

1. A method for identifying a collection of polymorphisms from nucleic acid molecules in a sample by analyzing a subset of the molecules, comprising the steps of:
- 5
- a. obtaining a nucleic acid-containing sample;
- b. treating the nucleic acid molecules in said sample to produce a reduced representation of nucleic acid fragments selected in a sequence-dependent manner by a method comprising:
- 10
- i. fractionating said nucleic acid molecules to produce nucleic acid fragments; and
- ii. selecting a subset of said nucleic acid fragments,
- wherein either (i) or (ii) or both (i) and (ii) are performed in a sequence-dependent manner;
- 15
- c. analyzing the reduced representation to identify pairs of fragments corresponding to the same chromosomal location, wherein fragments corresponding to the same chromosomal location are orthologous sequences; and
- d. comparing pairs of orthologous sequences to identify polymorphisms
- 20
- between said sequences.
2. The method of Claim 1, wherein the polymorphisms are single nucleotide polymorphisms.
3. The method of Claim 1, wherein the nucleic acid-containing sample is pooled from more than one individual.

4. The method of Claim 1, wherein the nucleic acid molecules are DNA.
5. The method of Claim 1, wherein the nucleic acid molecules are RNA.
6. The method of Claim 3, wherein the individuals share a particular trait.
7. The method of Claim 6, where the trait is a disorder.
- 5 8. The method of Claim 1, wherein step (b)(i) is performed by one or more restriction endonucleases.
9. The method of Claim 8, wherein the one or more restriction endonucleases are selected from the group consisting of *Bgl*III, *Xho*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, and *Hae*III.
- 10 10. The method of Claim 1, wherein step (b)(ii) is performed using an agarose gel.
11. The method of Claim 1, wherein step (b)(ii) is performed using high pressure liquid chromatography (HPLC).
12. The method of Claim 1, wherein step (b)(ii) is performed by selecting nucleic acid fragments which hybridize to selected additional nucleic acid sequences.
- 15 13. The method of Claim 1, wherein step (c) and/or step (d) are performed by determining at least a portion of the nucleic acid sequence of the orthologous sequences.

14. A method for identifying a collection of polymorphisms from nucleic acid molecules in a sample by analyzing a subset of the molecules, comprising the steps of:
- a. obtaining a nucleic acid-containing sample to be assessed;
  - 5 b. treating nucleic acid molecules in said sample to produce a reduced representation of nucleic acid fragments selected in a sequence-dependent manner by a method comprising:
    - i. fractionating said nucleic acid molecules with one or more restriction endonucleases to produce nucleic acid fragments; and
    - 10 ii. selecting a subset of said nucleic acid fragments using size fractionation;
 wherein either (i) or (ii) or both (i) and (ii) are performed in a sequence-dependent manner;
  - 15 c. analyzing the reduced representation to identify pairs of fragments corresponding to the same chromosomal location, wherein fragments corresponding to the same chromosomal location are orthologous sequences; and
  - d. comparing pairs of orthologous sequences to identify polymorphisms between said orthologous sequences,
  - 20 thereby identifying a collection of polymorphisms from said nucleic acid molecules.
15. The method of Claim 14, wherein the polymorphisms are single nucleotide polymorphisms.
16. The method of Claim 14, wherein the nucleic acid-containing sample is pooled  
25 from more than one individual.
17. The method of Claim 14, wherein the nucleic acid molecules are DNA.

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18. The method of Claim 14, wherein the nucleic acid molecules are RNA.
19. The method of Claim 16, wherein the individuals share a particular trait.
20. The method of Claim 19, wherein the trait is a disorder.
21. The method of Claim 14, wherein the one or more restriction endonucleases are  
5 selected from the group consisting of *Bgl*III, *Xho*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, and *Hae*III.
22. The method of Claim 14, wherein step (b)(ii) is performed using an agarose gel.
23. The method of Claim 14, wherein step (b)(ii) is performed using high pressure liquid chromatography (HPLC).
- 10 24. The method of Claim 14, wherein step (b)(ii) is performed by selecting nucleic acid fragments which hybridize to selected additional nucleic acid sequences.
25. The method of Claim 14, wherein step (c) and/or step (d) are performed by determining at least a portion of the nucleic acid sequence of the orthologous sequences.
- 15 26. The method of Claim 14, wherein the one or more restriction endonucleases cleave DNA on average about once every 2000 base pairs.
27. The method of Claim 14, wherein the subset of (b)(ii) is in a size range selected from the group consisting of: from about 380 base pairs to about 480 base pairs, from about 400 base pairs to about 500 base pairs, from about 480 base pairs to

about 580 base pairs, from about 500 base pairs to about 600 base pairs, and from about 540 base pairs to about 640 base pairs.

28. A method for genotyping a nucleic acid sample for polymorphisms in nucleic acid fragments contained in a reduced representation, comprising the steps of:
- 5 a. obtaining a nucleic acid-containing sample;
- b. treating the nucleic acid molecules in said sample to produce a reduced representation of nucleic acid fragments selected in a sequence-dependent manner by a method comprising:
- 10 i. fractionating said nucleic acid molecules to produce nucleic acid fragments; and
- ii. selecting a subset of said nucleic acid fragments, wherein either (i) or (ii) or both (i) and (ii) are performed in a sequence-dependent manner; and
- 15 c. analyzing the nucleic acid fragments contained in the reduced representation to assess the genotype at one or more polymorphic sites.
29. The method of Claim 28, wherein step (b)(ii) is performed using an agarose gel.
30. The method of Claim 28, wherein step (b)(ii) is performed using high pressure liquid chromatography (HPLC).
31. The method of Claim 28, wherein step (b)(ii) is performed by selecting nucleic acid fragments which hybridize to selected additional nucleic acid sequences.
- 20 32. The method of Claim 28, wherein step (c) is performed by determining at least a portion of the nucleic acid sequence of the nucleic acid fragments.

33. The method of Claim 28, wherein step (c) is performed by attaching specific oligonucleotide linker sequences to the fragments in the reduced representation and then amplifying said fragments.
34. The method of Claim 33, wherein the amplification is performed by polymerase chain reaction using primers complementary to the linker sequences.
35. The method of Claim 33, wherein the amplification is performed by cloning the fragments in an organism.
36. The method of Claim 28, wherein step (c) is performed by performing single-base extension reactions on the reduced representation.
37. The method of Claim 33, wherein step (c) is performed by performing single-base extension reactions on the reduced representation.
38. The method of Claim 28, wherein step (c) is performed by hybridization to an oligonucleotide array.
39. The method of Claim 33, wherein step (c) is performed by hybridization to an oligonucleotide array.
40. The method of Claim 28, wherein step (c) is performed by an oligo ligation assay.
41. The method of Claim 33, wherein step (c) is performed by an oligo ligation assay.

42. The method of Claim 1, wherein step (c) is performed by the following steps:
- a. comparing the sequences of the two members of a proposed pair,  
wherein the two sequences are further analyzed if the two sequences are  
at least 80% identical over at least 80% of the length of the shorter of the  
two sequences;
  - b. aligning the two sequences identified from (a), wherein the two  
sequences are further analyzed if the two sequences are identical over 10  
or more bases within the first 50 bases and the last 50 bases of the  
sequences;
  - c. identifying candidate single nucleotide polymorphisms in the sequences  
of (b), wherein the two sequences are further analyzed if the number of  
candidate single nucleotide polymorphisms does not exceed 1% of the  
total number of bases in the shorter of the two sequences, wherein two  
sequences which meet the criteria of (a) - (c) qualify as a candidate  
match;
  - d. repeating (a) - (c) for all proposed pairs; and
  - e. determining the number of candidate matches for the same chromosomal  
location, wherein said candidate matches are accepted if said number of  
matches does not exceed expectations,
- wherein accepted candidate matches are considered a pair.
43. The method of Claim 42, wherein said expectations are determined according to  
binomial or Poisson distributions.

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44. The method of Claim 14, wherein step (c) is performed by the following steps:
- a. comparing the sequences of the two members of a proposed pair,  
wherein the two sequences are further analyzed if the two sequences are  
at least 80% identical over at least 80% of the length of the shorter of the  
two sequences;
  - b. aligning the two sequences identified from (a), wherein the two  
sequences are further analyzed if the two sequences are identical over 10  
or more bases within the first 50 bases or the last 50 bases of the  
sequences;
  - c. identifying candidate single nucleotide polymorphisms in the sequences  
of (b), wherein the two sequences are further analyzed if the number of  
candidate single nucleotide polymorphisms does not exceed 1% of the  
total number of bases in the shorter of the two sequences, wherein two  
sequences which meet the criteria of (a) - (c) qualify as a candidate  
match;
  - d. repeating (a) - (c) for all proposed pairs; and
  - e. determining the number of candidate matches for the same chromosomal  
location, wherein said candidate matches are accepted if said number of  
matches does not exceed expectations,
- wherein accepted candidate matches are considered a pair.
45. The method of Claim 44, wherein said expectations are determined according to  
binomial or Poisson distributions.

46. A method for determining a limited population of polymorphisms from nucleic acid molecules in a sample, comprising the steps of:
- a. obtaining a nucleic acid-containing sample to be assessed;
  - b. treating nucleic acid molecules in said sample to produce nucleic acid fragments selected in a sequence-dependent manner by a method comprising:
    - i. fractionating said nucleic acid molecules to produce nucleic acid fragments; and
    - ii. selecting a subset of said nucleic acid fragments;wherein either (i) or (ii) or both (i) and (ii) are done in a sequence-dependent manner;
  - c. selecting from said subset nucleic acid fragments which occur at a corresponding chromosomal locus, thereby producing a pair, and
  - d. identifying polymorphisms between fragments of a pair;
- thereby determining a limited population of polymorphisms from said nucleic acid-containing sample.
47. A method for determining a limited population of polymorphisms from nucleic acid molecules in a sample, comprising the steps of:
- a. obtaining a nucleic acid-containing sample to be assessed;
  - b. treating nucleic acid molecules in said sample to produce nucleic acid fragments selected in a sequence-dependent manner by a method comprising:
    - i. fractionating said nucleic acid molecules with one or more restriction endonucleases to produce nucleic acid fragments; and
    - ii. selecting a subset of said nucleic acid fragments using size fractionation;wherein either (i) or (ii) or both (i) and (ii) are done in a sequence-dependent manner;

- 5                   c.       selecting from said subset nucleic acid fragments which occur at a  
                      corresponding chromosomal locus, thereby producing a pair, and  
                      d.       identifying polymorphisms between fragments of a pair;  
                      thereby determining a limited population of polymorphisms from said nucleic  
                      acid-containing sample.
48.   A method for genotyping a nucleic acid-containing sample from an individual  
      for polymorphisms, the method comprising:
- 10           a.       obtaining a first nucleic acid-containing sample to be assessed;  
              b.       treating nucleic acid molecules in said sample to produce a reduced  
                      representation of nucleic acid fragments selected in a sequence-  
                      dependent manner by a method comprising:
- i.       fractionating said nucleic acid molecules to produce nucleic acid  
                      fragments; and  
              ii.       selecting a subset of said nucleic acid fragments;
- 15           wherein either (i) or (ii) or both (i) and (ii) are done in a sequence-  
                      dependent manner;
- c.       analyzing the reduced representation to identify pairs of fragments  
                      corresponding to the same chromosomal location, wherein fragments  
                      corresponding to the same chromosomal location are orthologous  
                      sequences;
- 20           d.       comparing pairs of orthologous sequences to identify polymorphisms  
                      between the orthologous sequences;
- e.       obtaining a second nucleic acid-containing sample from an individual to  
                      be assessed; and
- 25           f.       analyzing said second nucleic acid-containing sample to assess the  
                      genotype at one or more polymorphisms identified in (d).

49. A method according to Claim 48, wherein the second nucleic acid-containing sample is treated by a method identical to step (b).

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11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040 1041 1042 1043 1044 10

## ABSTRACT OF THE DISCLOSURE

Novel methods of reproducibly determining a limited population of  
5 polymorphisms are disclosed.

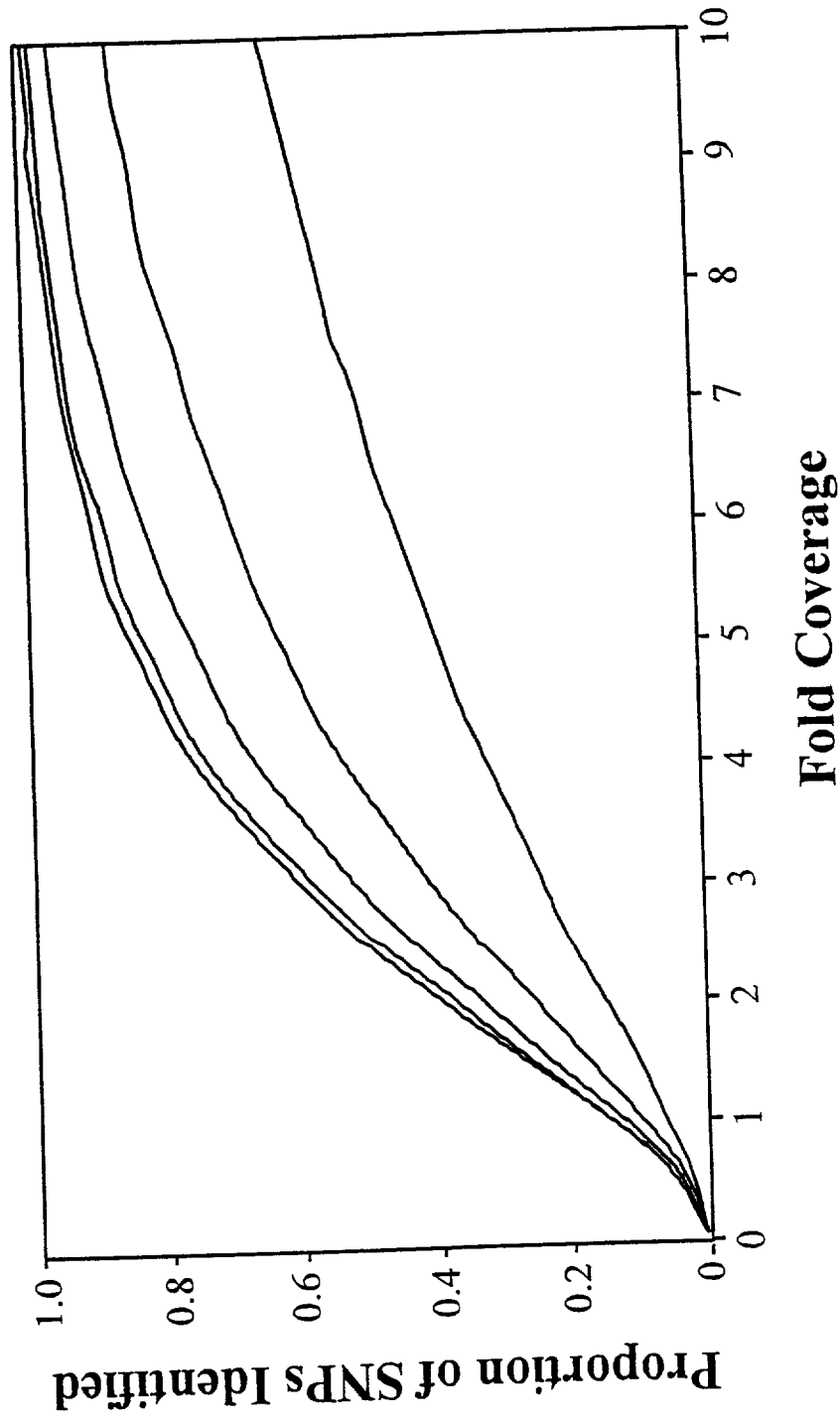


FIG. 1

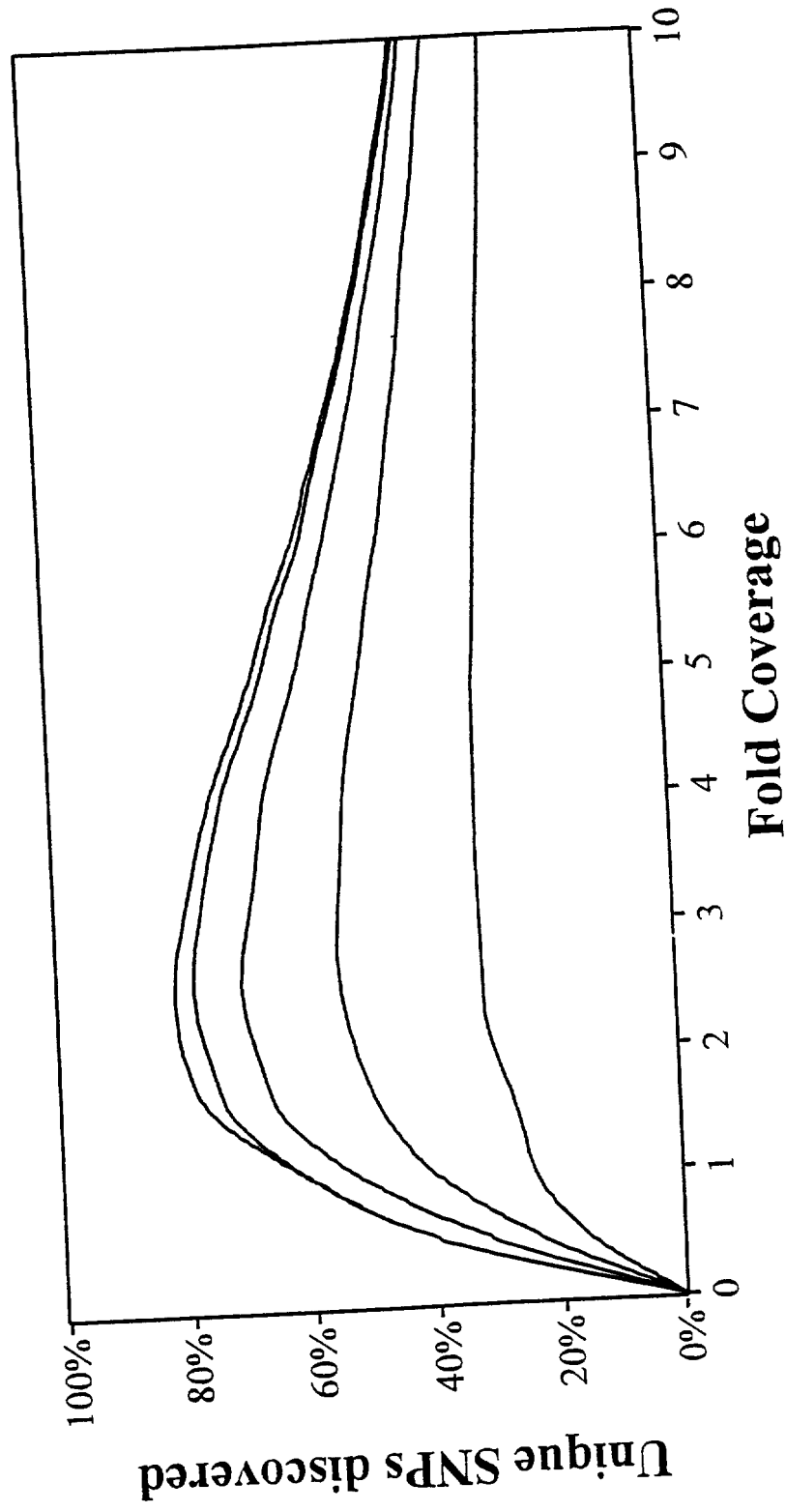


FIG. 2

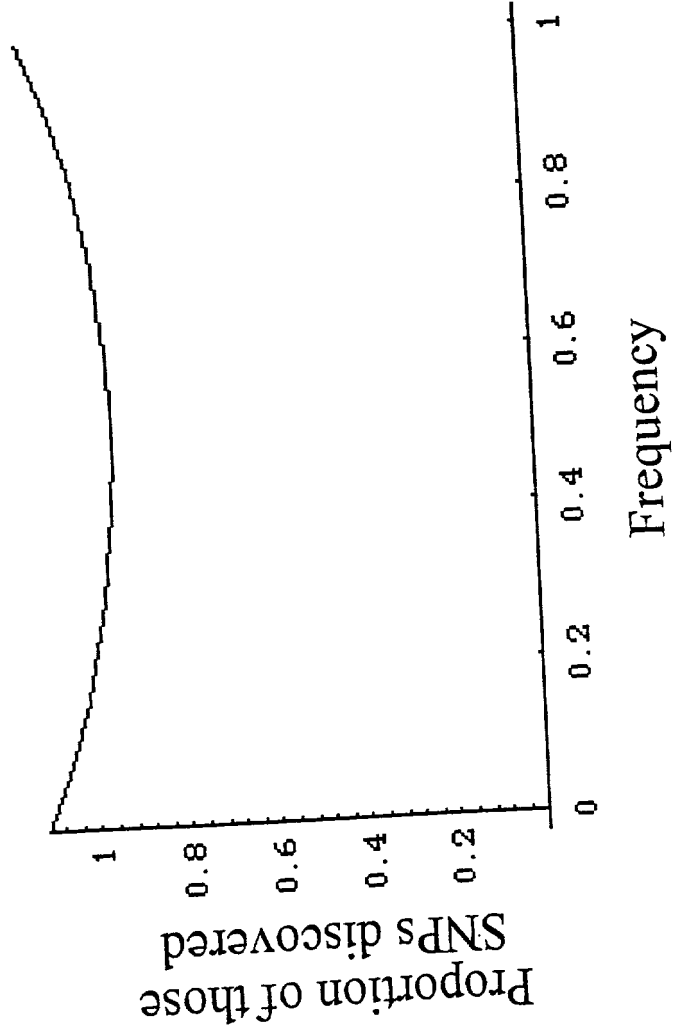


FIG. 3

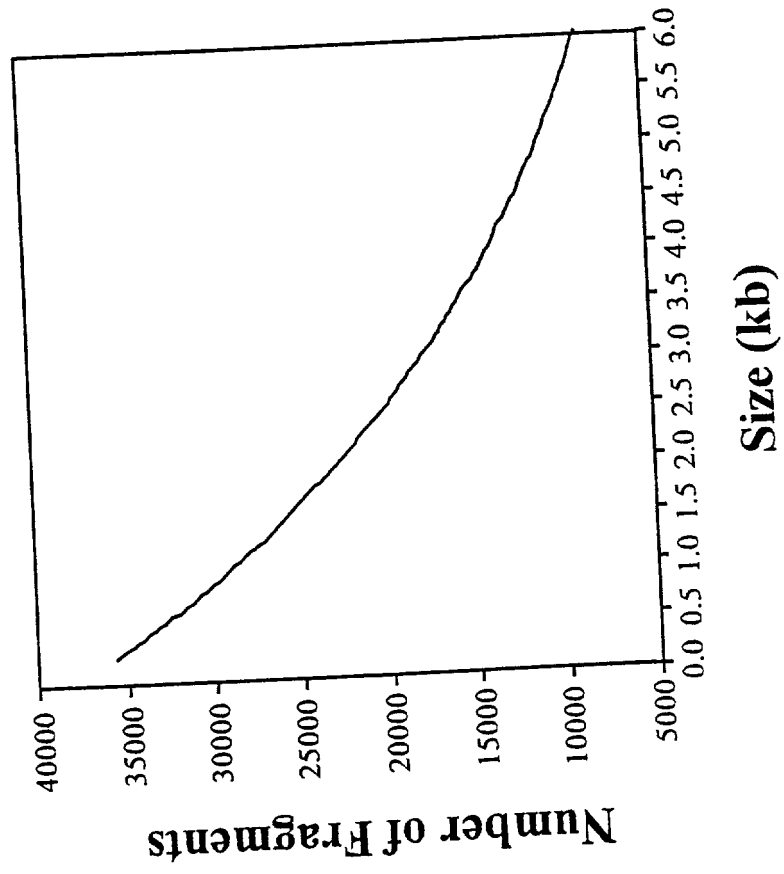


FIG. 4

FIG. 5

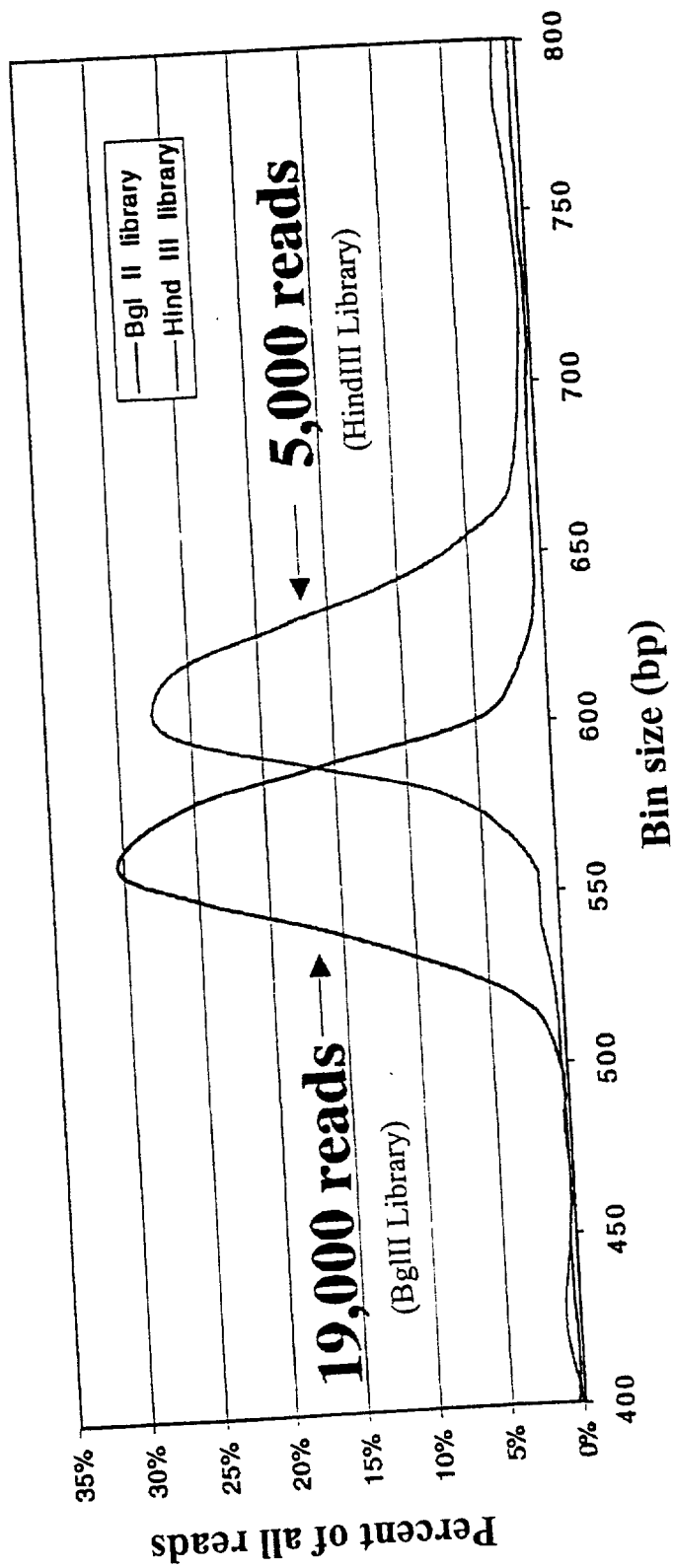
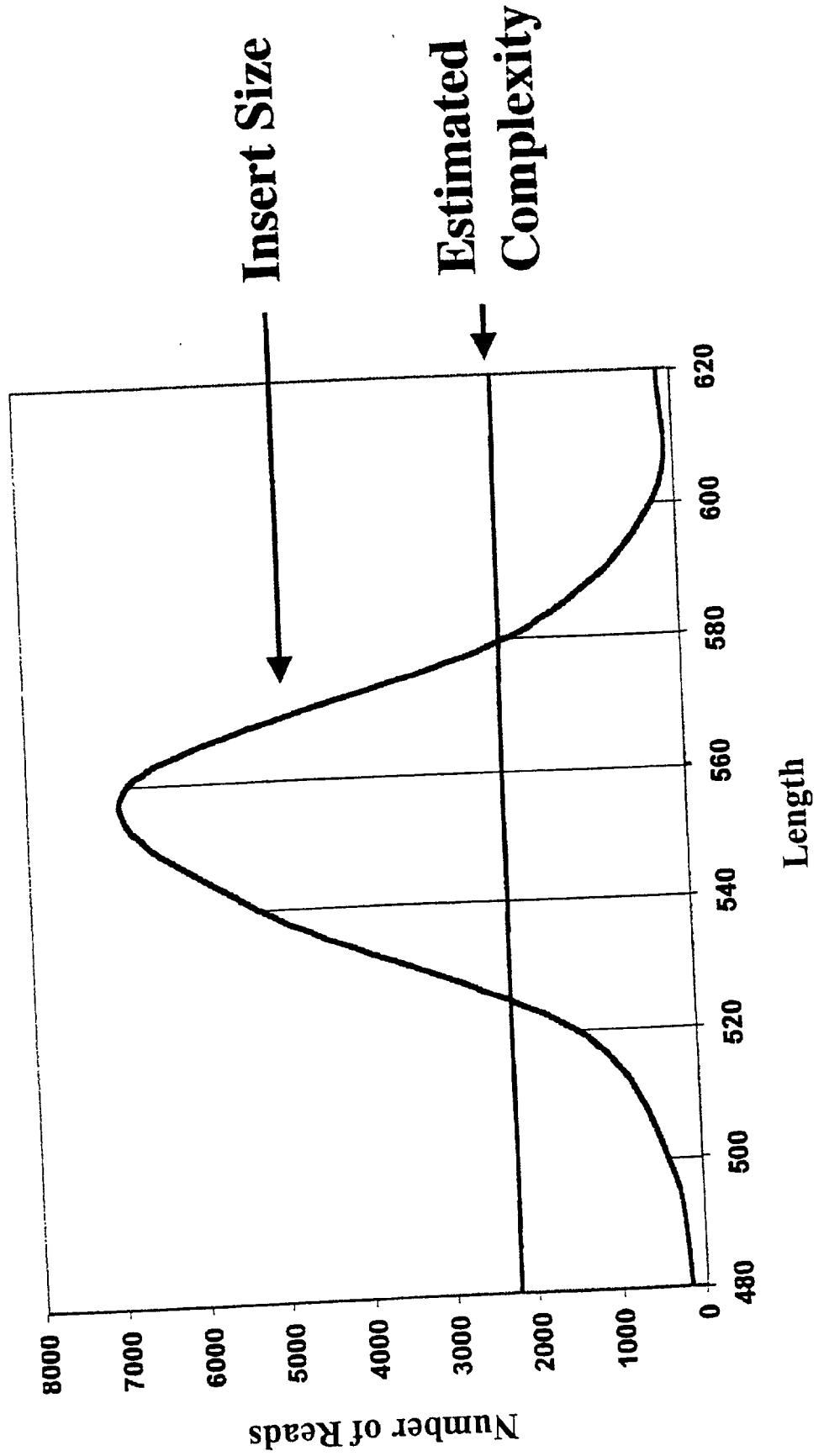
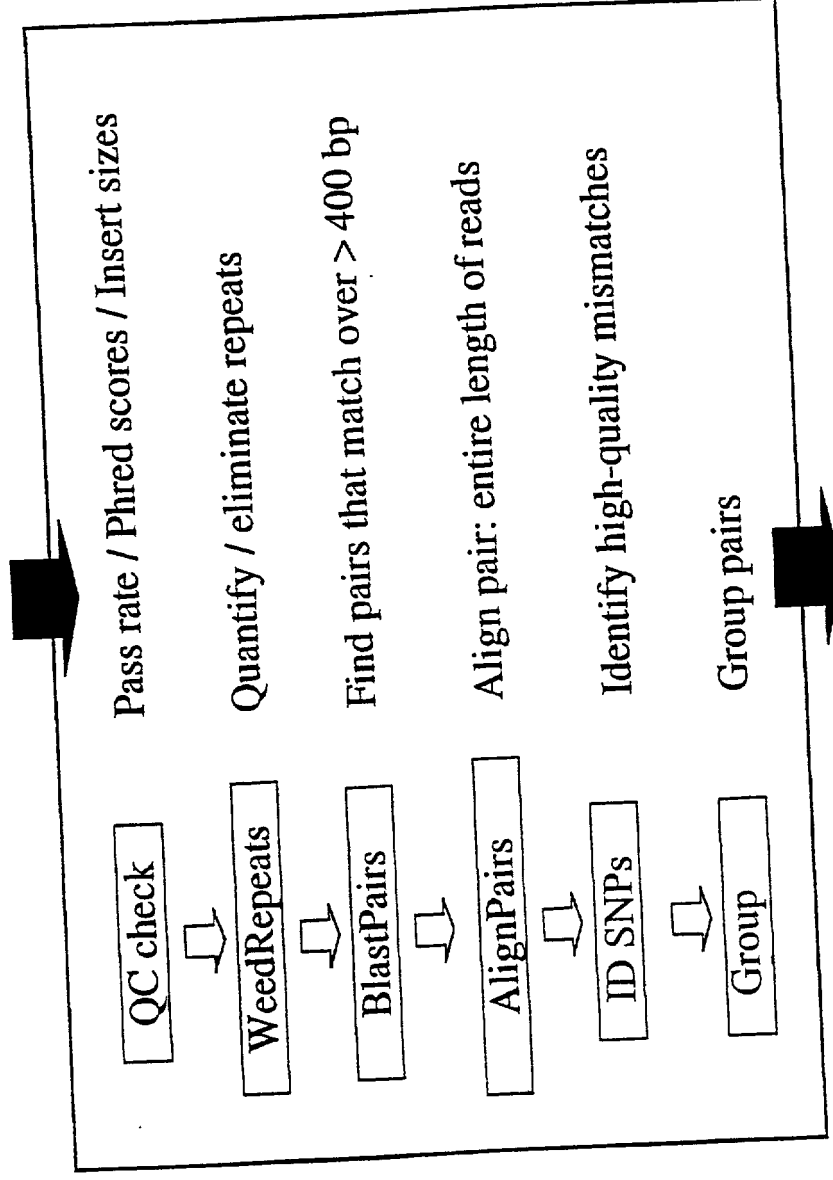


FIG. 6



# Raw Sequence



SNPs

FIG. 7



FIG. 8

